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WHEY UTILIZATION—GROWTH CONDITIONS FOR *SACCHAROMYCES FRAGILIS**

By Aaron E. Wasserman, William J. Hopkins, and Nandor Porges

Eastern Regional Research Laboratory,† Philadelphia, Pa.

Whey, a by-product of the cheese industry, is produced at the rate of more than 12 billion pounds annually (1), of which approximately 75 per cent is disposed of as waste. Containing 4.8 per cent lactose and 0.85 per cent protein (2)(3), the discarded whey represents a reservoir of approximately 450 million pounds of sugar and 80 million pounds of protein. Conversion of this reserve into utilizable products would relieve many plants and municipalities of a serious waste disposal problem.

Conversion of whey into yeast for the supplementation of animal diets has been considered (4)(5)(6), since such yeast contains thiamin, riboflavin, and ascorbic acid (7)(8)(9), while the protein is nutritionally similar to high-grade plant protein (5). *Saccharomyces fragilis* has been grown on whey (10), and in Germany *Torula* species have been grown in plants operating in conjunction with dairies (11). Several patents have been issued in the United States for the growth of yeast on whey, principally for their enzymes (12)(13)(14) and vitamins (7). However, large-scale production of yeast from whey has not been carried out extensively. Reductions in the production costs might make this process an attractive method for the utilization of whey.

The preliminary laboratory experiments reported herein indicate that large quantities of yeast may be produced from raw whey in 3 to 4 hr.

Materials and Methods

S. fragilis, NRRL Y-1109, used previously (6) to produce solids from whey, was carried on slants of yeast-maltose agar (15). However, the actual seed for an experiment was obtained from the harvested cell paste of a previous experiment. The fermentors were glass tubes, 12 in. long and 3½ in. in diameter, closed by two aluminum plates. A strong vortex was imparted to the liquid in each fermentor by an impeller rotating at 3,000 rpm and powered by a 1/30-hp motor. Air was supplied at the rate of 2 liters per minute per fermentor through an opening in the bottom plate centered under the impeller. The whey, obtained from a local cheese manufacturer, contained 4.0 per cent lactose, approximately 0.55 per cent lactic acid, and 0.09 per cent organic nitrogen. Fermentation runs were made with 500-ml quantities of the unsterilized whey media at a temperature maintained between 28 and 30°C.

Cell counts are reported as the average of two determinations made with a hemocytometer. The limitations of the cell count method are recognized, but this procedure is useful for rapid analysis of the course of yeast cell increase.

Dry weight determinations were made by centrifuging 5 ml of the yeast-medium suspension, washing the yeast with distilled water, centrifuging again, and drying at 100°C overnight. The results are reported as net increase in dry weight.

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† A laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

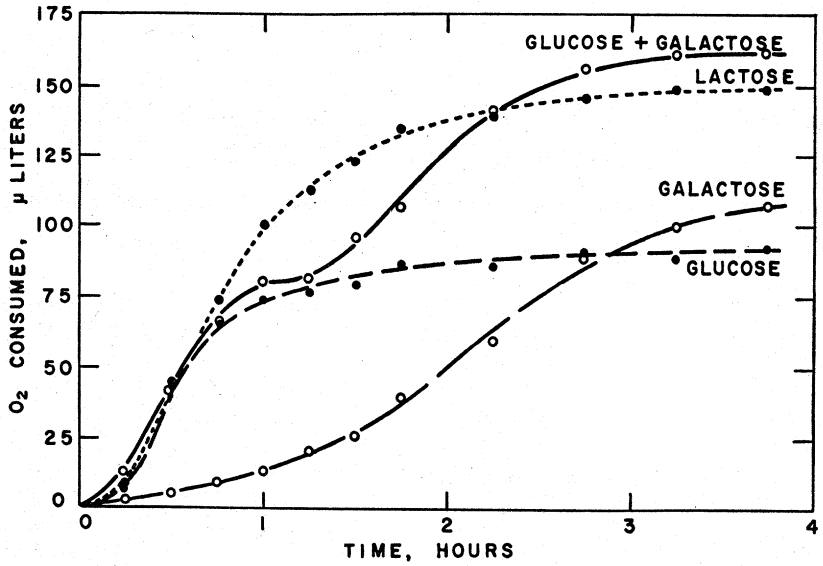

FIGURE 1.—Oxidation of sugars by *Saccharomyces fragilis* in Warburg studies.

Lactose determinations were made by the method of Stiles, Peterson, and Fred (16), and nitrogen by a modification of the Koch-McMeekin method (17). Lactic acid was determined by the Barker and Summerson method (18). Paper chromatography of sugars was conducted in butanol : acetic acid : water (4:1:5), and developed sheets were sprayed with triphenyl tetrazolium chloride reagent (19).

The data presented are representative of many experiments.

Manometric Studies

Preliminary studies on the utilization of lactose and its component sugars by *S. fragilis* were carried out in the Warburg respirometer (Figure 1). Galactose was oxidized more slowly than glucose, and the course of oxidation appeared to be adaptive. In a mixture of the two sugars, glucose was preferentially dissimilated, followed by galactose, giving a diauxie type curve (20). With lactose as substrate, a smooth oxygen consumption curve was obtained. These data indicate that lactose is utilized directly without prior hydrolysis. Further confirmation was obtained by chromatographic analyses of the media from fermentor experiments where the only sugar observed was lactose. The absence of glucose in the media from the fermentors was also shown by negative tests with "Clinisticks," a qualitative test specific for glucose and sensitive to 0.05 per cent of the sugar (21). The data confirm the "direct fermentation" of lactose by yeast reported by other workers (15)(22)(23).

Complete oxidation to carbon dioxide and water of the 500 mg of lactose used would require 396 μl of oxygen; however, only approximately 35 per cent of this quantity of oxygen was utilized, and CO_2 determinations also showed the production of approximately 36 per cent of the total CO_2 anticipated. Therefore, 65 per cent of the carbon of lactose was available for assimilation and, under ideal conditions, a yeast yield equivalent to 65 per cent of the carbon content of the sugar could be expected.

Fermentor Studies

Nitrogen and Phosphorus

The effects of nitrogen and phosphorus additions to whey are shown in Table I. Experiments were conducted

TABLE I.—Effect of Nitrogen and Phosphorus on Yeast Yield from Whey*

Substrate	Cell Count ($\times 10^6$/ml)	Net Dry Wt (mg/ml)	Lactose Used (mg/ml)
Whey	1,650	10.64	4.4
Whey + 1% $(NH_4)_2SO_4$	1,350	9.08	6.4
Whey + 1% K_2HPO_4	1,480	12.14	7.2
Whey + 0.25% each salt	1,430	12.96	10.2
Whey + 0.5% each salt	2,480	16.14	19.16
Whey + 1.0% each salt	2,400	16.50	20.60

* Initial yeast count 500 × 10^6/ml; growth period 6 hr.

with the medium initially adjusted with H_2SO_4 to pH 4.6, seeded to contain 500 × 10^6 cells per ml, and allowed to progress for 6 hr. In the fermentor containing whey alone, a pH change from 4.6 to 8.0 was observed at the end of 6 hr. Addition of $(NH_4)_2SO_4$ caused little change in the yeast yield or lactose utilization, although the final pH was not as great as in the whey alone. One per cent K_2HPO_4 added to the whey resulted in a small increase in the yeast yield, but more important, the pH rose only to approximately 6.4 in 4 hr, then decreased again to the range of 4.5 to 5.0. The values for all measured parameters of growth increased when both salts were added to the medium, and the optimal concentration appeared to be 0.5 per cent of each salt. The pH changes were similar to those observed with K_2HPO_4 alone.

Complete utilization of the lactose in the 6-hr fermentation period was not attained, although considerably more sugar disappeared in the presence of 0.5 per cent quantities of both salts than in their absence, or in the presence of each salt alone.

It was noticed in these and subsequent experiments that during the early stages of growth, the dry weight cell accretion was considerably greater than could be calculated from the lactose utilization. However, when sugar disappearance was stimulated by the addition of salts and other factors, the ratio of net yield to sugar utilization was nearer to calculated amounts. Some experimental data, not reported here, indicate that if the slow lactose utilizing fermentations were allowed to proceed 24 hr instead of 6 hr, the sugar was completely utilized and the net yield of cells was low compared to the sugar disappearance.

The factors involved in the growth of the cells in the early stages of the fermentation have not been investigated at this time; however, two possible suggestions are offered to account for the apparently high yield of cells:

1. Under the conditions of the experiment, the yeast are using other carbon sources in addition, or in preference, to the lactose (lactic acid and organic nitrogen-containing compounds are readily used by the yeast, as will be reported later).

2. Some compound reacting in the non-specific reducing sugar assay for lactose may be excreted by the yeast in the early stages of growth.

Yeast Extract

The effect of yeast extract on the growth of *S. fragilis* was investigated. Yeast cells grown through several transfers on the whey medium (whey + 0.5 per cent $(NH_4)_2SO_4$ + 0.5 per cent

TABLE II.—Effect of Yeast Extract on Yeast Yield from Whey*

Substrate	Cell Count ($\times 10^6$/ml)	Net Dry Wt (mg/ml)	Lactose Used (mg/ml)
Whey medium without yeast extract	1,400	14.60	24.0
Whey medium with yeast extract	3,070	21.22	40.0

* Seed yeast grown on medium minus yeast extract; initial count 500 × 10^6/ml; growth period 6 hr.

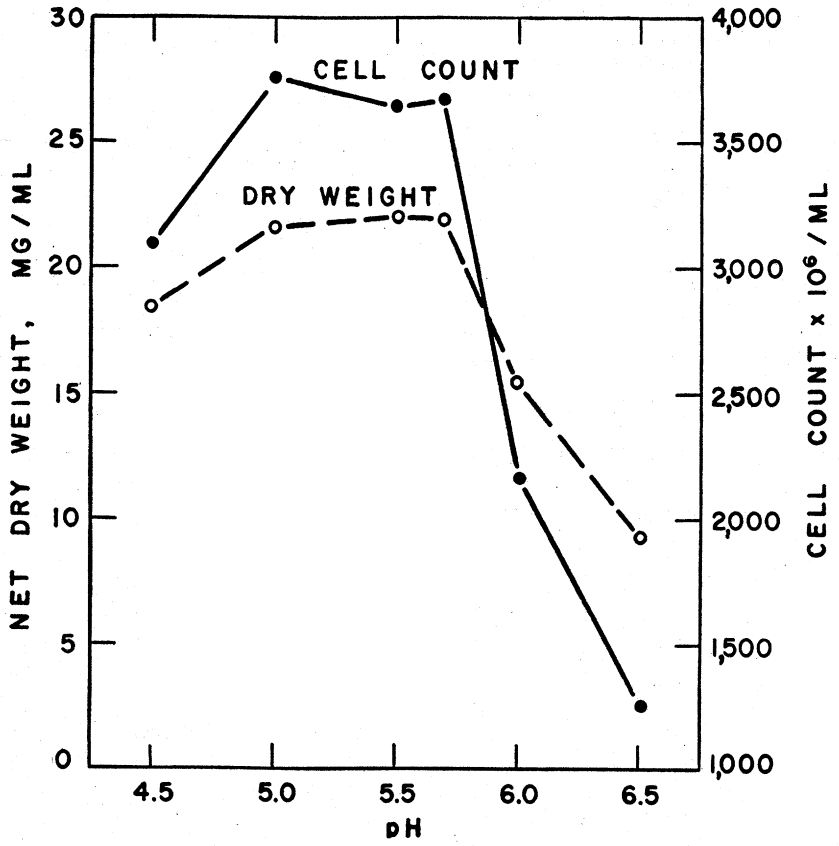

FIGURE 2.—Effect of initial pH of medium on the cell count and net dry weight of *Saccharomyces fragilis*.

K_2HPO_4) were divided equally between two fermentors—one containing the whey medium, and the second containing the whey medium + 0.1 per cent Difco yeast extract. The assimilation was allowed to proceed for 6 hr. A great stimulatory effect of the yeast extract was noted on the cell count, the net dry weight, and sugar utilization (Table II). Further studies on the optimal concentration of yeast extract were not carried out, although test tube growth studies indicated that 0.05 per cent yeast extract was also stimulatory to yeast growth.

The effect of the yeast extract is undoubtedly not due to the additional carbon or nitrogen, since the quantity is so small. Therefore, some vitamin or growth factor must be present. Apparently this factor is accumulated by the yeast grown in yeast extract-containing media, since it was found that transferring such yeast into whey medium with added salts, but lacking the yeast extract, resulted in a good yield for at least one transfer, before dropping back to the smaller yields obtained previously.

Although other organic extracts or concentrates have not been tested at this time, it is possible the growth factor may be found in other materials.

pH

The experiments described heretofore were carried out with the medium adjusted initially to pH 4.6. It was noted that in the medium containing both salts, the pH rose to 6.0–6.5, then became more acidic again. It was decided, therefore, to investigate the effect

of the initial pH on the yield of *S. fragilis*. Media, containing whey + 0.5 per cent each of both salts + 0.1 per cent yeast extract, were adjusted to various pH levels and seeded with approximately the same amount of seed. The fermentations were allowed to proceed for 7 hr. The growth response leveled off between pH 5.0 and pH 5.7 (Figure 2) with lower yields in cell count and net dry weight on either side of these values. It was decided to adopt an initial pH of 5.7 for the whey medium. This was the pH attained on addition of 0.5 per cent $(NH_4)_2SO_4$ and 0.5 per cent K_2HPO_4. Furthermore, the medium seemed more stabilized at this pH, with the occurrence of only very small changes in acidity or alkalinity. Thus, maintenance of pH did not become a problem during yeast growth. It was interesting to note that at an initial pH of 6.0 and 6.5, the medium became alkaline to approximately 8.0 to 8.5 as the yeast grew, and strong ammoniacal odors were observed.

Inoculum Size

The previous experiments were carried out with an inoculum size of approximately 500 million cells per ml (equivalent to about 4 mg dry wt). However, occasionally when larger inocula were used accidentally, it was noted that greater amounts of sugar were used in the 6-hr growth period. It was decided, therefore, to assess the influence of inoculum size on the yield of yeast (Table III). As the inoculum size increased from 300 million to 2.1 billion cells per ml, the time required for complete removal of the sugar dropped from over 6 hr to 3 hr. The maximum number of cells, approximately 4.5 billion, was attained in 3 to 4 hr with the larger inocula, while longer periods of time were required with the smaller quantities of seed. This also held true for the gains in net dry weights of the yeast cells, an average of about 23 mg/ml being the maximum attainable yield.

TABLE III.—Influence of Inoculum Size on Yield of Yeast

Inoculum Size ($\times 10^6$/ml)	Cell Count ($\times 10^6$/ml)	Net Dry Wt (mg/ml)	Lactose Used (mg/ml)
300	2,520	19.40	27 in 6 hr
500	3,100	20.86	31 in 6 hr
725	2,835	17.10	19 in 4 hr
	4,140	25.06	40 in 6 hr
1,165	3,650	20.38	30 in 4 hr
	4,880	25.38	40 in 5 hr
1,720	4,560	22.84	40 in 4 hr
2,140	4,520	23.52	40 in 3 hr

TABLE IV.—Yield of Yeast from Deproteinized and Raw Whey*

Medium	Cell Count ($\times 10^6$/ml)	Net Dry Wt (mg/ml)	Lactose Used (mg/ml)
Deproteinized whey	4,755	25.18	40
Raw whey	5,100	27.00	40

* Initial yeast count, 1,000 $\times$ 10^6/ml; growth period 6 hr.

Heat Deproteinization

Literature reports (3) (10) (24) have indicated that sterilization of the whey and removal of the heat-precipitated protein leads to growth of yeast superior to that attained in untreated whey. Since the yields obtained in these experiments were substantial, it was of interest to compare the effect of sterilizing the whey. Accordingly, whey at pH 4.5 was heated at 121°C for 15 min, filtered, and supplemented with the usual salts and yeast extract. The gains in net dry weight of the yeast and the sugar utilization were similar in both raw and deproteinized whey (Table IV).

Nitrogen Metabolism

Since the utilization of nitrogen is an important aspect in the growth of

yeast, the availability of nitrogen in the whey medium was investigated. With whey alone, yeast growth involved the disappearance of approximately 35 per cent of the whey nitrogen, which is almost entirely in the organic fraction. Since the organic nitrogen fraction contains amino acids, peptides, and other nitrogenous compounds, as well as protein, it was of interest to determine the pattern of utilization for growth. The supernatant from an experiment using whey + 0.5 per cent salts was assayed for inorganic nitrogen and total nitrogen, the difference between the two being the organic nitrogen fraction. Protein was precipitated with 15 per cent trichloracetic acid and the supernatant analyzed for non-protein nitrogen (NPN). The results in Table V show the pattern of nitrogen utilization obtained in several experiments.

Studies reported on the nitrogen metabolism of brewer's yeast showed that the amino acids in the medium were principally deaminated and then decarboxylated (25)(26). The ammonia, although shown as being free, was probably transferred to another acceptor to form an assimilable nitrogen compound. The alcohol resulting from this reaction was considered a member of the "fusel oil" fraction. A like process probably occurred during the growth of the *S. fragilis* on whey. During the first 1 to 2 hr, corresponding to the period of highest organic nitrogen utilization, the medium has a very fruity ester odor, characteristic of amyl or iso-amyl alcohol.

TABLE V.—Changes in Nitrogen of Supernatant Solution During Growth of Yeast on Whey + 0.5 Per Cent $(NH_4)_2SO_4$

Time* (hr)	Tot. N (mg/ml)	Inorg. N (mg/ml)	Org. N (mg/ml)	Prot. N (mg/ml)	NPN† (mg/ml)
0	1.95	1.05	0.90	0.57	0.33
2	1.45	0.87	0.58	0.22	0.36
4	1.16	0.48	0.68	0.28	0.40
6	1.0	0.32	0.68	0.28	0.40

* Initial yeast count, 400 × 10^6/ml; final count, 2,200 × 10^6/ml.
† Non-protein nitrogen.

Under conditions evoking a change in the yeast metabolism (such as an initial medium pH of 6.5), the amino group, instead of being transferred to an acceptor, may be released as NH_3 by a mechanism like the Stickland reaction (27).

Lactic Acid

Approximately 0.55 per cent lactic acid was found in the whey, as a result of the cheese manufacturing process. The lactic acid was used very rapidly by the yeast and disappeared in the early stages of the growth.

Discussion

Since economic factors are important considerations in the growth of yeast on whey, decreased processing costs would tend to increase the commercial feasibility of this operation.

The length of time required to produce the maximum net weight of yeast has been reduced to 3 to 4 hr from the 12- to 24-hr operations generally reported. Inoculation with large quantities of yeast (up to one-half the final number) leads to a rapid consumption of the whey lactose. The net increase in yeast weight in 3 to 4 hr is as great as the increased weight attained with smaller inocula in longer periods of time. The composition of the yeast cells may be different under these conditions, but data concerning this point are not yet available.

Sterilization of the whey and removal of the precipitated protein was unnecessary. In these short term experiments, little difference was observed between the yields of yeast grown on raw or deproteinized wheys. The problem of contamination was minimized by the large inoculum and the short duration of the growth period. The presence of the lactic acid bacteria in the raw whey (a residual from the cheese starter cul-

ture) may actually be a valuable adjunct in converting lactose into more readily utilized lactic acid. Graham *et al.* (4) have reported that lactose-fermenting bacterial contaminants speeded up the growth process of several yeast strains. The rapid growth of large quantities of yeast is a highly aerobic process, and the proper supply of oxygen is necessary. A study leading to calculations for the oxygen requirements of yeast growth in whey is presently under way. Some theoretical calculations have been made at this time to define the yeast obtainable from the lactose and lactic acid present in whey.

In these studies 40.0 mg lactose + 5.5 mg lactate in 1 ml whey contained 18.2 mg carbon; of the carbon 1/3 was converted to $CO_2 = 6.06$ mg carbon; therefore, 2/3 of the carbon was available for assimilation = 12.14 mg carbon.

If the dry yeast cell contains 45 per cent carbon (28), the 12.14 mg carbon should yield 27 mg yeast per ml whey. The average yeast yield obtained approximated 23 mg/ml, averaging 85 per cent of the theoretical yield.

Summary

Whey, a by-product of cheese processing, can be used as substrate for the growth of *Saccharomyces fragilis*. The addition of 0.5 per cent $(NH_4)_2SO_4$, 0.5 per cent K_2HPO_4, and 0.1 per cent yeast extract are necessary for good growth. Maximum growth is attained when the medium is initially adjusted to pH 5.0 to 5.7. Seeding with a high inoculum (about 2×10^9 yeast cells/ml) reduces the length of growing period to 3 to 4 hr. The yeast grow as well in non-sterile whey as in a sterile, deproteinized whey. The nitrogen metabolism is discussed.

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